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13. ABSTRACT *(Maximum 200 words)*

Angiogenesis determines tumor progression and microvessel density correlates with metastatic potential of breast cancer. Expression of endothelial cell receptors, Tie1 and Tie2, was described in areas of active vascular remodeling. There is no information on the role of Tie receptors in breast cancer progression. We selected breast cancer tissues and normal specimens and analyzed a fraction of these samples for Tie expression. We found differences in mRNA content in malignant and benign breast tumors. These exciting observations suggest that Tie receptors play an important role in angiogenesis associated with breast cancer progression. We also established that expression of Tie1 and 2 in human endothelial cells is induced by cell-cell interaction and is highest in adult quiescent endothelium. We will establish whether the frequency and pattern of expression of Tie 1 and 2 in breast cancer differ from expression in adjacent tissue and normal breast tissue. Statistical correlation with tumor progression will be obtained following analysis of clinical data. These studies will determine whether Tie1 and Tie2 are novel markers of angiogenic potential of breast cancer.

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Introduction

There is growing evidence that **tumor-associated angiogenesis** plays a fundamental role in tumor progression. The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Folkman and colleagues over two decades ago (1). Their work documented the extent to which tumor development depends on neovascularization and suggested that this relationship involves angiogenic growth factors which are specific for neoplasms. Vascular Endothelial Growth Factor, (**VEGF**) and its specific receptors on endothelial cells (EC), **Flt-1** (2) and **Flk-1/KDR** (3), modulate endothelial cell (EC) proliferation. VEGF is induced by hypoxia in areas of new blood vessel formation and healing wounds.

Recently, two homologous EC-specific receptors have been found to play a major role in vascular system development. **Tie 1 and 2** (Tyrosine kinase receptors with Immunoglobulin and EGF homology domains) are a novel subfamily of the PDGF receptor family (4, 5). The transcripts of Tie 2 and Tie 1 are detected sequentially at one-half day intervals and follow expression of VEGF receptors. The common features of mice which lack Tie 1 or Tie 2 are extensive hemorrhages, defective vascular branching and capillary development resulting in embryonic death (6-8).. **Therefore, Tie 1 and 2 receptors are essential for angiogenesis and integrity of vascular EC whereas VEGF mediates early hemangioblast differentiation and vasculogenesis in the embryo.**

In contrast to evidence that VEGF expression is upregulated by hypoxia and activation of VEGF receptors by therapeutic administration of VEGF enhances neovascularization in an adult animal, the role of Tie receptors in the postnatal period is not clear. Activation of Tie2 by a recently discovered ligand, Angiopoietin 1, does not induce EC proliferation. Expression of Tie1 and 2 declines after birth but their mRNAs and proteins are detected in normal and tumor-associated EC (9-11). Although VEGF is expressed in breast cancer (12), correlation between its expression and invasive potential of breast cancer is poor (13, 14). Microvessel density correlates with tumor progression in breast cancer (15-17) and with the invasive potential of ductal adenocarcinoma (18). Tie 1 mRNA was identified in metastatic melanoma and breast cancer (19, 20). In addition, Tie 2 antagonist, a soluble form of Tie 2 receptor, induced regression of a mouse mammary tumor *in vivo* (21).

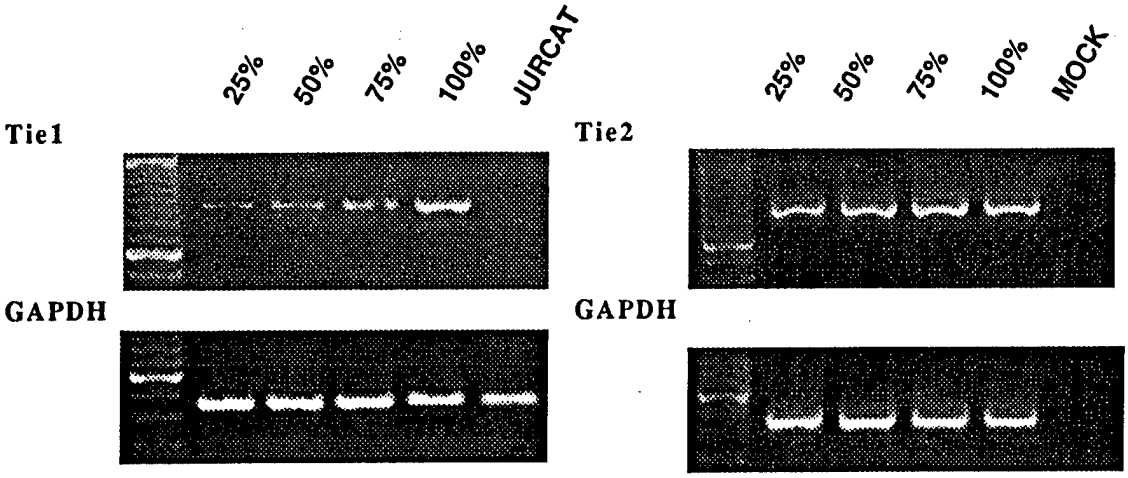
These discoveries suggest that expression of Tie 1 and 2 gene products mark the development of tumor-associated angiogenesis. Our central hypothesis is that expression of EC Tie receptors in breast cancer predicts tumor vascularization and correlates positively with invasive and metastatic potential of early stage breast cancer.

Body of Work

To determine the frequency and pattern of expression of Tie receptors in metastatic breast disease, we collected surgical specimens of cancer and noninvolved normal breast from 30 patients with breast cancer. To date, nine samples have been analyzed. RNA from normal human lung was used as a positive control. Preliminary studies reveal substantially higher levels of Tie 2 in metastatic lesions as compared with normal tissue or morphologically early breast cancer. Results of Tie1 mRNA levels did not significantly differ between normal and malignant breast tissue.

To determine whether expression of Tie 1 or 2 occurs in early stages of breast cancer, we collected 12 samples and anticipate obtaining an additional 5-8 within the next two months. Obtaining these samples was difficult because many were needle biopsies and were used for diagnostic pathology. Samples will be analyzed simultaneously when collection is completed. Correlation of expression of Tie with metastatic potential is ongoing through analysis of patients' records (ie, diagnosis, course, treatment, development of metastasis, etc.).

We determined whether expression of Tie 1 and 2 in EC is modulated by cell density. Human umbilical vein EC (HUVEC) were grown to confluency, trypsinized and replated at 25, 50, 75 and 100% confluency. Fourteen hours later, cells were harvested and RNA was extracted. RT-PCR was performed for Tie1, Tie2 and GAPDH as a control for the loading of RNA. Cells plated at confluency had higher levels of mRNA for Tie. These results suggest that Tie expression may be induced by cell-cell interaction. Therefore, mature tumor-associated vessels may contain higher levels of Tie gene products. These studies were confirmed by immunohistochemistry of Tie expression in EC of normal and denuded artery during EC repair (9).



The following methods were used or developed for use in these studies:

RNA isolation

RNA from normal human tissues and cell lines was extracted using ULTRASPEC™-II RNA isolation system (Biotecx Laboratories, Houston, TX). Briefly, Ultraspec RNA solution was added to either homogenized tissue samples or cultured cells followed by extraction with chloroform and phenol/chloroform/isoamyl alcohol. RNA was then precipitated with isopropanol, purified with RNATack resin and eluted with DEPC treated water.

RNA Concentration

The concentration of purified RNA was estimated by measuring its absorbance at 260nm in the spectrophotometer. 1OD unit at 260nm corresponding to 40μg/ml of total RNA was used to calculate the concentration.

Concentration of total RNA = (OD_{260nm} x 40μg/ml) x dilution.

The purity of RNA was estimated by A_{260}/A_{280} ratio. The expected ratio of pure RNA is 1.7-2.0.

Normal Tissues

100mg of frozen human placenta tissue was grinded on ice using Proscientific tissue grinder (probe of 7mm) followed by RNA isolation as described above.

Cell Lines

- Human Umbilical Vein Endothelial Cells (HUVEC);
- Hybrid of HUVEC and adenocarcinoma of lung (EAhy 926) (28);
- Human Umbilical Microvascular Endothelial Cells (HUMEC);
- Human Breast Adenocarcinoma Cell Line (MDA MB468) (29);
- Human Breast Adenocarcinoma Cell Line, pleural effusion (MCF7) (30);
- Mouse Fibroblast Cell Line, immortalized (NIH 3T3);
- EAhy 926 expressing Ang-2 gene product (31);
- EAhy 926 expressing vector alone as a negative control (31).

Briefly, cells were grown in 100mm^2 dishes to 80-90% confluency and the media was removed by suctioning. Cells were then lysed directly in the plates with 1ml Ultraspec solution, scrapped on ice into microcentrifuge tubes and RNA was extracted as described above.

Reverse Transcription-Polymerase Chain Reaction

Reverse Transcription Reaction (RT) was performed using SuperScript II RNaseH Reverse Transcriptase Kit (Gibco BRL, Life Technologies, Rockville, MD). Briefly, 1-5μg of total RNA in a RNase-free tube was mixed with 1μl of Oligo dT primers and DEPC treated water to a final volume of

12μl. The reaction was incubated at 70°C for 10min, chilled on ice and briefly spun down. The pre-mix (4μl 5X RT buffer, 2μl 0.1M DTT, 1μl 10mM dNTPs mix (Promega) and 1μl SuperScript II Reverse Transcriptase) was added to the reaction and the sample was incubated at 42°C for 50-60min, at 70°C for 15min and then chilled on ice. The final product was diluted with 80μl of sterile water and stored at -20°C.

Polymerase Chain Reaction (PCR) conditions were optimized for each of the specific primers and are as follows:

- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers; human, rat, mouse compatible (Clontech Laboratories, Palo Alto, CA). Product length - 450bp.

  5' ACCACAGTCCATGCCATCAC 3'

  5' TCCACCACCCGTTTGCTGTA 3'

PCR reaction mixture was adjusted to a final volume of 50 μl containing 10% of total RT reaction product, PCR buffer (1X corresponding to 60mM Tris-HCl, 15mM ammonium sulfate, pH8.5, Invitrogen), 2.5mM dNTPs (each), Taq DNA polymerase in storage buffer B (1Unit at 5U/μl, Promega), two specific primers (250ng each at 100ng/μl) and $MgCl_2$ as 1.5mM Hot Wax Beads (Invitrogen). The samples were denatured (94°C, 1min), annealed (52°C, 30sec) and extended (72°C, 1min30sec) for 30-40 cycles.

- TIE-1 primers, product length - 276bp.

  5' CTCAGGGACCTTGACACTTACCCG 3'

  5' AGGAGGTTGATGATGTTGGGGTGA 3'

PCR reaction mixture was adjusted to a final volume of 50 μl containing 10% of total RT reaction product, PCR buffer (1X corresponding to 10mM Tris-HCl, pH8.3, 50mM KCl, Perkin Elmer), AmpliTaq DNA polymerase (1Unit at 5U/μl, Perkin Elmer), 1.5mM $MgCl_2$ (Perkin Elmer), 2.5mM dNTPs (each) and two specific primers (250ng each at 100ng/μl). PCR was performed under the same conditions as described for GAPDH primers.

- TIE-2 primers, product length 264bp.

  5' AGTAGCCATATTCACCATCCACCG 3'

  5' TGCCAAGCCTCATAGTGATTAACG 3'

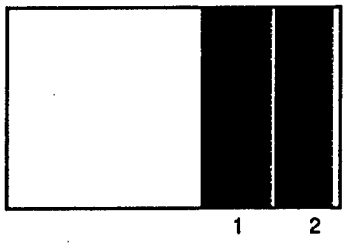
PCR reaction mixture was prepared as for TIE-1 primers. The cycle conditions were as described for GAPDH primers except the annealing temperature was changed to 55°C.

Selection of Breast tissue samples

9 random tissue samples were retrieved from the Pathology Department, SEMC where they have been stored at -80°C.

Preparation of the Samples

Breast tissue samples selected for the analysis were cut as shown below to generate two adjacent pieces: #1 was used for preparation of paraffin embedded slides for future experiments and #2 was used for RNA extraction, RT-PCR and PCR. The rest of the samples were stored at -80°C in Pathology Department, at SEMC.



RNA Extraction/RT-PCR

RNA extraction and RT-PCR from breast tissue samples were performed as described above. Due to the extremely low RNA concentration from each sample, accurate estimation of RNA volume required for the reaction was not possible. Therefore an equal volume of each sample was used in the RT reaction (11μl). PCR results were then normalized to an internal control (GAPDH).

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords (22), and grown as previously reported (23). EA.hy 926, a hybrid cell line obtained by fusion of HUVEC and A549 human lung carcinoma cell line, was a gift from Dr. C-J S. Edgell (24) and cultured in Dulbecco's modification of Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS) to confluency on 0.5% gelatin (Sigma) coated culture dishes. HUVECs or EA.hy 926 cells were plated at 25%, 50%, 75%, and 100% confluence. After overnight incubation, cells were harvested to extract total RNA. Jurkat a human T-cell leukemia cell line was cultured in RPMI containing 10% FBS to 2-4x10^6 cells/ml and harvested as above.

Conclusions

The results from the initial year of studies indicate that Tie1 and 2 are expressed in adult endothelial vessels and their expression is increased in breast cancer lesions. These exciting observations suggest that Tie 1 and 2 play an important role in angiogenesis associated with breast cancer progression. We also established that expression of Tie1 and 2 in human endothelial cells is induced by cell-cell interaction and is highest in adult quiescent endothelium. We are in the process of establishing whether the frequency and pattern of expression of Tie 1 and 2 in primary and metastatic breast cancer differ from expression in adjacent tissue and normal breast tissue. Statistical correlation with tumor progression will be obtained following analysis of clinical data. These studies will determine whether Tie1 and Tie2 are novel markers of angiogenic potential of breast cancer.

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